



Baculovirus expression, biochemical characterization and organophosphate sensitivity of rBmAChE1, rBmAChE2, and rBmAChE3 of *Rhipicephalus (Boophilus) microplus*

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ABSTRACT

Rhipicephalus (Boophilus) microplus cDNAs, *BmAChE1*, *BmAChE2*, and *BmAChE3*, were previously identified as presumptively encoding acetylcholinesterases (AChEs), but biochemical identity was confirmed only for recombinant *BmAChE3*. In the present study, four recombinant *BmAChE1* constructs and single recombinant constructs of *BmAChE2* and *BmAChE3* were expressed in baculovirus. Biochemical characterization of the recombinant proteins supports classification of rBmAChE1, rBmAChE2, and rBmAChE3 as AChEs (E.C.3.1.1.7), as evidenced by (i) substrate preference for acetylthiocholine, (ii) inhibition by eserine, BW284c51, and the organophosphates (OPs) malaoxon and paraoxon, (iii) insensitivity to iso-OMPA, and (iv) rapid hydrolysis of acetyl- β -methyl-thiocholine. Unlike reports for insect AChEs, we did not observe substrate inhibition of activity at acetylthiocholine concentrations as high as 40 mM, however, product inhibition was apparent at 10–100 μ M choline in agreement with properties reported for the catalytic domain of *Anopheles gambiae* acetylcholinesterase-1. Substrate affinity and V_{\max} values were highest for rBmAChE1 proteins, and one rBmAChE1 enzyme (Tx11, derived from the OP-resistant strain Tuxpan), was insensitive to paraoxon and exhibited a greatly reduced V_{\max} near that of rBmAChE2. To date, recombinant *BmAChE1* and *BmAChE3* enzymes with reduced sensitivity to OP-inhibition have been cloned and expressed from OP-resistant strains. The presence of at least three genes expressing AChEs in *R. (B.) microplus*, at least two of which contain mutations expressed as OP-insensitive enzymes, strongly suggests that phenotypic resistance to OPs may be complex and multigenic in character.

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1. Introduction

The Southern cattle tick, *Rhipicephalus (Boophilus) microplus*, a vector of bovine babesiosis and anaplasmosis, was eradicated from the United States (Graham and Hourigan, 1977), but remains endemic to Mexico. Re-establishment of *R. microplus* within the United States is prevented by the Cattle Fever Tick Eradication Program (CFTEP) including a permanent quarantine zone along the

southern border of Texas maintained by the Veterinary Services branch of the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture. All cattle imported from Mexico are required to be dipped in vats containing the organophosphate (OP), coumaphos (George, 1996). The CFTEP is considered to be in a near crisis condition with concerns of increasing OP resistance in Mexico (Santamaría and Fragoso, 1994; Fragoso et al., 1995), increasing numbers of tick outbreaks in the counties adjacent to the border with Mexico prompting temporary expansion of the quarantine area by nearly a million acres in July of 2008, the recent finding of an OP-resistant outbreak strain within the U.S. quarantine zone (Miller

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et al., 2005), and fears regarding the potential failure of the United States entry barrier to *R. microplus* (Davey et al., 2003; Temeyer et al., 2004b). Acetylcholinesterase (AChE, EC 3.1.1.7) is the target for OP acaricides (O'Brien, 1967), and it was reported that AChE insensitivity is the primary mechanism of OP-resistance in *R. microplus* (Lee and Batham, 1966; Schuntner et al., 1968; Bull and Ahrens, 1988; Li et al., 2003), although metabolic detoxification may also be important (Li et al., 2003; Rosario-Cruz et al., 2009). Elucidation of OP-resistance mechanisms at the molecular level in *R. microplus* is important for development of rapid diagnostic tests for resistance detection and choice of control options, for development of resistance management options, and for potential development of targeted control technology (Pang, 2006, 2007; Carlier et al., 2008; Pang et al., 2009; Rosario-Cruz et al., 2009).

Vertebrate AChE has been characterized as a single gene encoding multiple AChE transcripts produced by alternative splicing events (Schumacher et al., 1988; Massoulié et al., 1998). In *Drosophila melanogaster*, a single *ace* gene has been characterized (Fournier et al., 1992), however, reports of other invertebrate AChEs have highlighted the existence of multiple genes encoding AChE (Combes et al., 2000; Villatte and Bachman, 2002; Xu et al., 2003; Temeyer et al., 2004a; Ilg et al., 2010). Because the cholinesterase gene family contains a number of related enzymes and structural proteins (Cygler et al., 1993; Cousin et al., 1997; Zimmerman and Soreq, 2006), gene identification based on moderate sequence similarity alone can be misleading (Naumoff et al., 2004; Nagy et al., 2008). In *R. microplus*, Baxter and Barker (1998) first identified a cDNA, *BmAChE1*, presumptively encoding AChE, and found no mutations within *BmAChE1* sequences from various tick strains that would account for OP-resistance. Baxter and Barker (1998) suggested that there may be another gene encoding AChE that was responsible for resistance, or that resistance may result from post-translational modifications of *BmAChE1*. Subsequently, Hernandez et al. (1999) and Temeyer et al. (2004a) identified two additional cDNAs that presumptively encoded AChE, *BmAChE2* and *BmAChE3*, respectively, suggesting that several genes may encode AChE in *R. microplus*. Temeyer et al. (2006) later reported baculovirus expression and biochemical characterization of rBmAChE3 that was similar in biochemical properties to native AChE extracted from tick synganglial tissue, described by Pruett and Pound (2006), and was the first report of a recombinant tick AChE with enzymatic activity. Searches for mutations associated with OP-resistance in *BmAChE1* (Baxter and Barker, 1998, 1999), *BmAChE2* (Hernandez et al., 1999; Baxter and Barker, 2002) and *BmAChE3* (Temeyer et al., 2004a, 2006, 2009) failed to reveal a clear association between any identified amino acid substitutions and OP-resistant phenotype; however, baculovirus expression of rBmAChE3 demonstrated reduced paraoxon sensitivity of rBmAChE3 containing a predicted R86Q substitution (Temeyer et al., 2007). The purpose of the current study was to express recombinant proteins encoded by *BmAChE1* and *BmAChE2* to determine their biochemical identity, testing the hypothesis of multiple AChE-encoding genes within *R. microplus*.

2. Materials and methods

2.1. Tick material

This study utilized *R. microplus* tick strains colonized at the Cattle Fever Tick Research Laboratory (CFTRL) in Edinburg, TX. The tick strains differed in their OP-resistance status (S = susceptible, R = resistant), Deutch (S), San Román (R), and Tuxpan (R). The Deutch (S) strain was obtained in 2001 from an outbreak 20 miles northwest of Laredo, in Webb County, TX. The Tuxpan (R) strain originated in 1981 near Tuxpan in the state of Veracruz, Mexico, and was obtained from the National Parasitology Laboratory in Jiutepec, Morelos, Mexico in 1994. The San Román (R) strain originated near Champoton, Campeche, Mexico, and was established at the CFTRL in 1998. Both the Tuxpan and San Román strains were maintained at the CFTRL with organophosphate selection (Li et al., 2003).

2.2. Cloning and sequencing

Total RNA was isolated from pooled larvae or individual synganglia from adult females of each strain as described previously (Temeyer et al., 2007). Oligo-dT₁₈V or gene-specific primers (Table 1) were utilized to direct synthesis of first-strand cDNA from RNA template using Superscript III Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Design of PCR primers was aided by Oligo version 6.71 software (Molecular Biology Insights, Inc., Cascade, CO).

Complete coding regions from *BmAChE1*, *BmAChE2*, and *BmAChE3* were amplified by high fidelity PCR from cDNA using PCR primers listed in Table 1, sequenced, and expressed in baculovirus vectors essentially as described previously (Temeyer et al., 2007). Individual experiments utilized *PfuUltra*TM (Stratagene, Santa Clara, CA), *iPROOF*TM (Bio-Rad Laboratories, Inc., Hercules, CA), or *JumpStart*TM AccuTaq Red LA (Sigma Chemical, St. Louis, MO) hotstart high fidelity DNA polymerases. Blunt-ended amplified cDNA was incubated with AmpliTaq[®] DNA polymerase (Applied Biosystems, Foster City, CA) to enable TOPO[®] TA ligation into the pCR4[®]-TOPO[®] vector (Invitrogen Corp., Carlsbad, CA), and transformed into Stbl 2TM or TOP-10 chemically competent *E. coli* according to the manufacturer's instructions. Transformants were screened by PCR, and plasmid DNAs were prepared using either the QIAprep[®] Spin Miniprep Kit (Qiagen, Valencia, CA) or the Fast Plasmid Isolation Kit (5 Prime, Inc., Gaithersburg, MD), followed by sequencing using BigDye[®] terminator on an ABI3130xl Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Amino acid sequence numbering is provided with respect to the translational initiation codon for each of the three *BmAChEs*.

2.3. Baculovirus expression

Recombinant expression clones were assembled, sequenced, and expressed in baculovirus infected Sf21 cell cultures as described previously (Temeyer et al., 2006,

Table 1Oligodeoxynucleotide primers used in PCR amplification and cloning of *BmAChE1*, *BmAChE2*, and *BmAChE3*.

Gene	Primer name	Sequence (5' → 3')	Clones ^a
<i>BmAChE1</i>	Aust-21U17	GCTACGCGGACCTTTCA	SR, Synganglia
<i>BmAChE1</i>	BmAChE1-1854L16	ACGAGCTCCCACAGTA	SR, Synganglia
<i>BmAChE1</i>	Aust-10U16	TTTCGCGCGGAGCTAC	Tx, Deutch
<i>BmAChE1</i>	BmAChE1-1873L16	GCTGGCTGCTTCGATG	Tx, Deutch
<i>BmAChE2</i>	BmAChE2-46U13	GCGCGCAAAAATG	SR6, SR9, SR12
<i>BmAChE2</i>	BmAChE2-1739L21	ACGCACACTTATTCAGTATAG	SR6, SR9, SR12
<i>BmAChE2</i>	BmAChE2-44U15	GCGCGCGCAAAAATG	SR, Synganglia
<i>BmAChE2</i>	BmAChE2-1739L23	ACACGCACACTTATTCAGTATAG	SR, Synganglia
<i>BmAChE3</i>	BmAChE3-150U13	GGGGAGCACGGTA	Deutch
<i>BmAChE3</i>	BmAChE3-2088L15	GCGAATCTCCATCT	Deutch

^a SR, San Román; Tx, Tuxpan; all synganglia were San Román.

2007). Baculovirus-cell culture supernatants expressing AChE activity were used for biochemical characterization.

2.4. Determination of K_m and V_{max} for rAChEs

The AChE activity was assayed in microplates using acetylthiocholine iodide (AcSch, Sigma) or butyrylthiocholine iodide (BuSch, Sigma) as substrates. Enzyme samples were diluted [SR4 (1:2), SR11 (1:2), and Deutch 5 (1:3)] with buffer (50 mM sodium phosphate, pH 7.5), and all other samples were assayed undiluted. The standard assay consisted of 5 μ l of enzyme and 195 μ l of substrate solution (0.5 mM AcSch and 0.32 mM DTNB [Sigma, 5,5'-dithiobis(2-nitrobenzoic acid)] in 50 mM NaPO₄ buffer, pH 7.5) run in duplicate (AcSch serial dilutions 480–7.5 μ M; BuSch 480–30 μ M). Because of low AChE activity for samples SR12 and Tx11, enzyme volume was increased to 10 μ l. Initial velocities (V_0 , ΔOD) were determined within the linear region of the reaction curve. The reaction was monitored at 405 nm with a Bio-Tek EL808 Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) for 28 min with readings every 2 min. The background reaction was subtracted by use of a substrate only control. Values for K_m and V_{max} were calculated with the aid of SigmaPlot Enzyme Kinetics Module 1.2 (Systat Software Inc., Richmond, CA) on the initial velocities (V_0).

2.5. Substrate preference for AcSch vs BuSch

Baculovirus expression supernatant for each of the rBmAChE preparations was incubated with 1 mM substrate, either AcSch or BuSch and cholinesterase activity was monitored for up to 1 h. The slope of the linear portion of the reaction curves was compared for each substrate, with a more positive slope indicating substrate preference, which we expressed as a ratio of the slopes for AcSch:BuSch.

2.6. Effect of high substrate concentration or eserine sulfate inhibition on rAChE activity

In order to determine the effect of a high concentration of AcSch on enzyme activity, assays were conducted as above for the determination of K_m and V_{max} values except that substrate was diluted from 4.0×10^{-2} M to 4.0×10^{-6} M by log dilution. The reaction was monitored

for 30 min with readings every 2 min. Initial velocities (V_0 , ΔOD) were determined within the linear region of the reaction curve.

Each recombinant enzyme sample was evaluated in the presence of the AChE-specific inhibitor eserine sulfate (Sigma) at dilutions ranging from 2.0×10^{-3} M to 2.0×10^{-6} M. Enzyme volumes and concentrations were as stated above. Matched volumes of diluted inhibitors were added to each well. Substrate (AcSch, 120 μ M, 180 μ l) was added to the enzyme inhibitor mix and the reaction was monitored for 1 h with 10 min reads, and compared to an uninhibited control for percent inhibition.

2.7. Paraoxon inhibition of rAChEs

In order to determine the sensitivity of each rAChE to inhibition by the OP, paraoxon, an assay was run using the same volume and concentration of enzyme that was used in the determination of kinetic parameters. To each enzyme was added 15 μ l of paraoxon, diluted in 50 mM sodium phosphate buffer, ranging in concentration from 1.0×10^{-5} M to 1×10^{-9} M. The reaction was initiated by immediately adding 180 μ l of AcSch substrate at a concentration of 120 μ M, and was read every 10 min for 30 min. The percent residual AChE activity for each dilution of paraoxon was calculated relative to an uninhibited control reaction. The natural log (ln) of the percent residual AChE activity was plotted against the negative log of the paraoxon concentration. Linear regression analysis of the data points yielded an equation used to calculate the concentration of paraoxon required to inhibit the enzyme to 50% of control activity within 30 min.

2.8. Comparative sensitivity to inhibitors

Sensitivity of recombinant enzymes, Deutch5 (rBmAChE1), SR12 (rBmAChE2), and SR-BC26 (rBmAChE3) were compared for the AChE-specific inhibitor BW284c51 (1,5-Bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide, Sigma), eserine, malaoxon (Sigma), paraoxon, and the BuChE-specific inhibitors iso-OMPA (tetraiso-propylpyrophosphoramidate, Sigma) and ethopropazine hydrochloride (Sigma). Relative sensitivity to inhibitors was tested by preincubation of each enzyme with test concentrations of inhibitor for 10 min followed by addition of AcSch substrate mix. The final volume of each reaction

Table 2

Biochemical analysis of recombinant BmAChE1, BmAChE2, and BmAChE3.

Expression construct/ volume in assay	Acetylthiocholine iodide			Butyrylthiocholine iodide			Paraoxon concentration ($\times 10^{-7}$ M)	
	V_{\max} (10^{-7} mol $\text{min}^{-1} \mu\text{l}^{-1}$)	K_m (μM)	V_{\max}/K_m	V_{\max} (10^{-7} mol $\text{min}^{-1} \mu\text{l}^{-1}$)	K_m (μM)	V_{\max}/K_m	90% inhibition in 30 min	Insensitivity ratio relative to SR _{BC26} ^a
rBmAChE1								
Deutch5/1.7 μl	9.05	4.25	2.13	0.33	1.42	0.235	6.02	2.11
SR4/2.5 μl	5.86	2.87	2.04	0.23	1.10	0.210	4.62	1.62
SR11/2.5 μl	6.02	7.59	0.79	0.37	32.21	0.012	4.45	1.56
Tx11/10 μl	0.21	5.20	0.04	0.09	320.28	3.0×10^{-4}	115	40.28
rBmAChE2								
SR12/10 μl	0.17	52.7	0.003	0.03	71.91	4.3×10^{-4}	5.88	2.06
rBmAChE3								
SR-BC26/5 μl	4.31	90.19	0.05	0.11	309.30	3.6×10^{-4}	2.85	1.00

^a SR-BC26 is used as a reference for comparative purposes because it was previously characterized [24] and corresponds to the *BmAChE3* reference sequence (GenBank accession no. [AY267337](#)).

was 300 μl containing 0.5 mM AcSch and 0.32 mM DTNB in 50 mM NaPO₄ buffer, pH 7.5, enzyme (2.5 μl rBmAChE1, 50 μl rBmAChE2, or 5 μl rBmAChE3), and test inhibitor. The microtiter plate assay was conducted at 23 °C and was monitored at 405 nm for 60 min on a SpectraMAX™250 plate reader (Molecular Devices, Sunnyvale, CA). Final concentrations of inhibitor resulting in 50% reduction in the activity without inhibitor were determined by probit regression analysis. Statistical significance ($p \leq 0.05$) was determined using ANOVA and least significant difference t -test for means comparison (StatPac version 3.0, StatPac, Inc., Bloomington, MN).

3. Results

3.1. Biochemical characterization of recombinant BmAChEs

Values obtained for biochemical kinetics of rBmAChE1, rBmAChE2, and rBmAChE3 proteins are presented in Table 2. The K_m values for rBmAChE1, rBmAChE2, and rBmAChE3 are quite different from one another with rBmAChE1 exhibiting the highest affinity for AcSch. All three enzymes exhibited substantially higher V_{\max}/K_m ratios for AcSch than for BuSch, indicating substrate preference for AcSch and confirmed by direct comparison of hydrolysis of 1 mM substrate (Supplementary Figs. 1–6). All rBmAChE expression products were inhibited by eserine sulfate, and were not inhibited at high concentrations (40 mM) of AcSch. V_{\max} values for rBmAChE1 and rBmAChE3 were similar to one another and substantially higher than the V_{\max} for rBmAChE2.

3.2. Recombinant BmAChE1

Four rBmAChE1 constructs encoding predicted amino acid substitutions (Table 3) compared to GenBank accession [CAA11702](#) were expressed in the baculovirus system. Susceptible strain construct Deutch 5 encoded 13 amino acid substitutions, all of which were frequently found among susceptible strain sequences available to us. Two San Román (OP-R) strain constructs (SR4, SR11) were

expressed, encoding 13 amino acid substitutions, 9 of which were common to both. One construct (Tx11) was expressed from the OP-R strain Tuxpan, encoding 17 amino acid substitutions. Of the 17 amino acid substitutions predicted for the expression product of Tx11, six were shared among all of the rBmAChE1 constructs (E60K, P78T, T219A, A260T, A349V, and R549H) and were found in high frequency among *BmAChE1* transcripts from pooled larvae and individual synganglial transcripts from both OP-S and OP-R strains. Predicted amino acid substitutions D188G, E196G, V331A, and F390S were encoded only by the Tx11 construct which produced an expression product that was insensitive to paraoxon inhibition in comparison to all of the other rBmAChE1 expression products (Table 2).

Table 3Predicted amino acid substitutions^a in rBmAChE1 proteins expressed in baculovirus.

Deutch 5 (S)	SR4 (R)	SR11 (R)	Tx11 (R)
E60K	E55G		
P78T	E60K	E60K	E60K
	P78T	P78T	P78T
	P157S	P157S	
T219A			D188G
E238G	T219A	T219A	E196G
A260T	A260T	A260T	T219A
			E238G
			A260T
			V331A
A349V	N333S	A349V	
	A349V	T362A	A349V
K364R			K364R
			F390S
M426V			M426V
T437A			T437A
	Q488R	Q488R	
		I493T	
R549H	R549H	R549H	R549H
E552Q			E552Q
	W571F	W571F	
T576A			T576A
N583D			N583D

^a Amino acid substitution nomenclature: E55G refers to the glutamic acid (E) at position 55 replaced by glycine (G).

As shown in Table 2, K_m values of the AChE catalytic activity expressed from each construct were similar, ranging from 2.872 μM to 7.588 μM . AcSch was the preferred substrate over BuSch, as indicated by comparison of the V_{\max}/K_m ratios for the two substrates and by direct comparison of hydrolysis of 1 mM AcSch vs BuSch (Supplementary Figs. 1–4). Expression products of Deutch 5, SR4, and SR11 had comparable V_{\max} values ranging from 5.863×10^{-7} mol/min/ μl to 9.046×10^{-7} mol/min/ μl . AChE catalytic activity expressed from Tx11 exhibited a much reduced rate of product formation with a V_{\max} of 0.212×10^{-7} mol/min/ μl . Sensitivity to the OP inhibitor paraoxon was similar for AChE activities expressed from Deutch 5, SR4, and SR11 with insensitivity ratios, relative to SR-BC26 (a rBmAChE3 comparative reference), of 2.112, 1.621, and 1.560, respectively. The AChE activity expressed from Tx11 was the least sensitive to paraoxon of the rBmAChE1 proteins evaluated, with an insensitivity ratio of 40.28 relative to SR-BC26.

3.3. Recombinant BmAChE2

The rBmAChE2 construct, SR12, was expressed in the baculovirus system. SR12 was isolated from an OP-R strain and encoded only one predicted amino acid substitution, L195P, (compared to GenBank accession **AF067771**) that was present in all BmAChE2 transcripts observed from OP-S and OP-R strains. AChE enzymatic activity expressed from SR12 was low with a V_{\max} of 0.165×10^{-7} mol/min/ μl and a higher K_m (52.7 μM) than the rBmAChE1 expressed proteins. AcSch was the preferred substrate over BuSch, as indicated by the V_{\max}/K_m and by direct comparison of hydrolysis of 1 mM AcSch vs BuSch (Table 2, Supplementary Fig. 5). The enzyme was comparable to the rBmAChE1 proteins expressed from Deutch 5, SR4, and SR11 with respect to paraoxon inhibition with an insensitivity ratio of 2.063 relative to SR-BC26 (Table 2).

3.4. Recombinant BmAChE3

We used SR-BC26 as a comparative reference corresponding to the BmAChE3 reference sequence (GenBank accession **AY267337**) reported by Temeyer et al. (2004a). Like rBmAChE1 and rBmAChE2 expression products, AcSch was the preferred substrate over BuSch as indicated by the V_{\max}/K_m and by direct comparison of hydrolysis of 1 mM AcSch vs BuSch (Supplementary Fig. 6). The SR-BC26 had the highest K_m value of all rBmAChE expression products at 90.19 μM . The V_{\max} value for SR-BC26 was comparable to rBmAChE1 proteins expressed from Deutch 5, SR4, and SR11 (Table 2).

Further characterization of rBmAChEs to various inhibitors was conducted for comparative purposes and to determine conformity to properties previously described for insect acetylcholinesterases (Toutant, 1989; Jiang et al., 2009). As shown in Table 4, each of the rBmAChEs utilized acetyl- β -methylthiocholine (AcMeSch) nearly as efficiently (75–90%) as acetylthiocholine. Each of the rBmAChEs was inhibited by the AChE-specific inhibitors eserine and BW284C51, as well as by paraoxon, and malaoxon. All three rBmAChEs exhibited approx. 1000-

fold or greater sensitivity to the AChE-specific inhibitor BW284C51 than to the relatively BuChE-specific inhibitors ethopromazine or iso-OMPA. In addition, all three rBmAChEs were slightly inhibited at moderate concentrations of choline.

4. Discussion

This work is the first characterization of rBmAChE1 and rBmAChE2, confirming their biochemical identity as functional AChEs encoded by BmAChE1 and BmAChE2. The rBmAChEs met all of the criteria set by Toutant (1989) for a “true” cholinesterase (AChE), except inhibition at high substrate concentrations. We tested AcSch as high as 40 mM, encountering interference by excessive background above that value, but note that substrate inhibition did not occur until higher than 40 mM substrate concentrations for some mutants of *D. melanogaster* (Stojan et al., 2004) or until 100 mM for *Torpedo californica* AChE (Colletier et al., 2006). In this regard, the expressed rBmAChE proteins exhibited properties similar to the catalytic domain of acetylcholinesterase-1 of *Anopheles gambiae* (Jiang et al., 2009) for which part or all of the apparent inhibition at high substrate concentrations may have actually resulted from product inhibition by choline accumulating in the reaction mix. Expressed proteins encoded by rBmAChE1, rBmAChE2, and rBmAChE3 transcripts were inhibited at choline concentrations in the micromolar range. Other AChEs encoded by insects show variable degrees of inhibition by excess substrate (Zhu and Clark, 1994; Gao and Zhu, 2001; Stojan et al., 2004; Jiang et al., 2009). The rBmAChE1 proteins shared comparable K_m values and, with the exception of that encoded by Tx11, had similar enzyme activities (Table 2). The AChE activity expressed from Tx11, was the only recombinant AChE in this study exhibiting significant insensitivity to paraoxon, and in terms of V_{\max} values, had approx. 3% of the activity of the other rBmAChE1 enzymes. The rBmAChE2 enzyme had a K_m value approx. 10.5 times greater than the K_m values observed for the rBmAChE1 enzymes (Deutch 5, SR4, and SR11), indicating lower substrate affinity. Activity of the rBmAChE2 enzyme (SR12) was also low, with only 2% of relative activity (V_{\max}) compared to rBmAChE1 or rBmAChE3. These biochemical data support the claim that these expressed proteins are AChE enzymes encoded by the respective transcripts and verify that together with BmAChE3, *R. microplus* expresses at least 3 separate, functional AChE enzymes.

Recombinant BmAChE1-Tx11 demonstrated approx 40-fold decreased sensitivity to OP-inhibition indicated by its OP-insensitivity ratio. The four amino acid substitutions within the expressed rBmAChE1-Tx11 sequence that differentiate it from the expressed rBmAChE1-SR4 and -SR11 OP-R constructs (D188G, E196G, V331A, and F390S) are of interest from the perspective of the role they play in the observed biochemical properties, inferring an association between at least some of these substitutions and OP-insensitivity. Alignment of the predicted amino acid sequences for BmAChE1, BmAChE2, and BmAChE3 with AChE of *T. californica* or *D. melanogaster* (presented in Temeyer et al., 2004a) similar to the alignment of selected vertebrate and insect AChEs reported by Harel

Table 4Inhibitor concentration producing specified reduction of rBmAChE activity.^a

rBmAChE Construct	Choline <i>I</i> ₅₀ (mM)	Choline <i>I</i> ₁₀ (mM)	Eserine <i>I</i> ₅₀ (nM)	Paraoxon <i>I</i> ₅₀ (nM)	BW284c51 <i>I</i> ₅₀ (nM)	Malaoxon <i>I</i> ₅₀ (nM)	Ethopromazine <i>I</i> ₅₀ (μM)	Iso-OMPA <i>I</i> ₅₀ (mM)
rBmAChE1	5 (a)	0.1 (a)	1 (a)	20 (a)	0.39 (a)	10 (a)	1.7 (a)	>1 (a)
rBmAChE2	10 (a)	0.1 (a)	250 (b)	17 (a)	0.15 (a)	20 (ab)	100 (b)	>1 (a)
rBmAChE3	5 (a)	0.01 (a)	150 (b)	20 (a)	2 (b)	50 (b)	230 (c)	>1 (a)

^a *I*₅₀ refers to 50% reduction in uninhibited enzyme activity, *I*₁₀ refers to 10% reduction in uninhibited enzyme activity, different letters in parentheses following inhibitor concentration indicate significant ($p \leq 0.05$) differences.

et al. (2000) reveals that the aspartate at position 188 (D188G) in BmAChE1 is fully conserved in BmAChE2 and BmAChE3. This residue corresponds to *T. californica* N131 or *D. melanogaster* N163 for which similarity is conserved throughout vertebrate and insect sequences (Harel et al., 2000), and is immediately adjacent to tyrosine Y130/Y162 (*T. californica*/*D. melanogaster*, respectively) lining the catalytic gorge. Interestingly, a semi-conservative substitution (*Drosophila* I161V) on the other side of the conserved tyrosine Y130/Y162 has been reported to generate insecticide resistance (Menozi et al., 2004) and is located at the bottom of the active site behind Trp83, the main component of the choline binding site (Shi et al., 2004). Similarly, the nonpolar phenylalanine at position 390 in BmAChE1 (F390S) is fully conserved in BmAChE2 and BmAChE3, corresponding to A336/F376 in *T. californica*/*D. melanogaster*, respectively. Sequence identity/similarity at this site is conserved within insect AChEs and also within vertebrate AChEs but not between insect and vertebrate AChEs (Harel et al., 2000). F390S is 2 amino acids away from a fully conserved tyrosine lining the catalytic gorge (Y334/Y374 in *T. californica*/*D. melanogaster*) known to change conformation upon binding inhibitors (Harel et al., 2000) and the change to serine, a smaller polar side chain, may be significant. The remaining two substitutions predicted in rBmAChE1-Tx11, E196G and V331A, do not involve substitution of residues fully conserved in BmAChE2 and BmAChE3. Instead, the E196G substitution of glycine for glutamate matches the glycines at corresponding positions in BmAChE2 and BmAChE3 (E140/G172 in *T. californica*/*D. melanogaster*), a site with conserved similarity only in insects (Harel et al., 2000), suggesting that the E196G substitution is unlikely to produce conformational changes resulting in OP-insensitivity, although changes in electrostatic charge contribution to the dipole moment may be important (Silman and Sussman, 2008). The V331A substitution encoded by rBmAChE1-Tx11 (valine in BmAChE2, leucine in BmAChE3) results in a smaller side chain without change in charge or polarity, a site (I275/S317 in *T. californica*/*D. melanogaster*) with sequence identity/similarity conserved only within insects or vertebrates, but not between insects and vertebrates (Harel et al., 2000). These data suggest that the D188G and/or the F390S substitutions in rBmAChE1-Tx11 are most likely responsible for the differences observed in the altered K_m for AcSch and reduced inhibition by paraoxon, possibly by narrowing of the passageway within the catalytic gorge or through “second shell” effects (Harel et al., 2000) on conformation of residues at the catalytic site or lining the catalytic gorge. Although these two mutations have not been previously demonstrated to produce OP-insensitivity in arthropods,

studies by Villatte et al. (2000) suggest a large number of mutations could have that effect.

The three AChEs of *R. microplus* exhibit different amino acid sequences (Temeyer et al., 2004a) and biochemical properties (Tables 2 and 4), most notable of which is the differing K_m for AcSch. Phylogenetic mapping of the three BmAChEs with the amino acid sequences of other arthropod AChEs strongly suggests that they are not closely related to one another or to insect AChEs (Nardi et al., 2008). Why would *R. microplus* need 3 different acetylcholinesterases? A number of other arthropods have 2 genes encoding AChE, and vertebrates have one gene encoding AChE and one encoding butyrylcholinesterase (Toutant, 1989; Bourguet et al., 1997; Darvesh et al., 2003; Kozaki et al., 2008; Nardi et al., 2008). The synaptic function of AChE is well documented, however, a large amount of evidence implicates AChE in other physiological functions, some of which do not appear to require catalytic capability (Paraoanu et al., 2007; Greenfield et al., 2008; Inestrosa et al., 2008; Jiang and Zhang, 2008; Johnson et al., 2008; Soreq, 2008; Abreu-Villaça et al., 2010). Acetylcholinesterase is an ancient enzyme, found in bacteria and plants as well as animals, suggesting it performs a variety of functions in addition to its synaptic role (Wessler et al., 1998, 2001; Horiuchi et al., 2003; Silman and Sussman, 2005; Paraoanu and Layer, 2008). In general, vertebrates produce different forms of AChE that provide different functions through alternative splicing of exons and formation of multimeric forms or complexes with other proteins and like butyrylcholinesterase, has been implicated in a number of human disorders (Giles, 1997; Darvesh et al., 2003; Inestrosa et al., 2008). The separation of AChE activity among three BmAChEs suggests a separation of structure and function that may be novel in tick AChE. It is interesting that the *Ixodes scapularis* genome contains paralogs to each of the three BmAChE genes (Janice P. VanZee and Catherine A. Hill, personal communication). The physiological roles played by the individual BmAChEs remain to be determined, however, elucidation of their individual roles may provide less complex model systems for study of structure-function relationships held in common with vertebrate AChEs. In addition, separation of AChE activity in *R. microplus* may present an opportunity for development of species-selective acaricides based on structural features unique to one or more of the BmAChEs.

In summary, we confirmed that BmAChE1, BmAChE2, and BmAChE3 encode three functional AChEs expressed in *R. microplus*, and report an OP-insensitive rBmAChE1, rBmAChE1-Tx11. We expressed recombinant forms of all three AChEs, and observed that they displayed different kinetic properties. These findings support the hypothesis

that BmAChE1, BmAChE2, and BmAChE3 may perform different functions *in vivo*, and the physiological role of each of the AChEs remains to be elucidated. To date, specific cDNAs of *BmAChE1* and *BmAChE3* cloned from OP-resistant ticks and expressed in the baculovirus system generate recombinant acetylcholinesterases that exhibit reduced sensitivity to OP-inhibition, confirming the presence of OP-target-insensitive mutations in at least two of the three *BmAChEs*. The presence of at least three biochemically confirmed AChEs, and mutations generating OP-insensitivity in at least two of the three AChEs, strongly suggest that the contribution of OP-insensitive acetylcholinesterases to phenotypic OP-resistance in *R. microplus* is complex and may be multigenic.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.vetpar.2010.04.016](https://doi.org/10.1016/j.vetpar.2010.04.016).

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